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—Original Article—

The transfer of parthenogenetic embryos following artificial insemination in cows can enhance pregnancy recognition via the secretion of interferon tau

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Abstract. Repeat breeding is a reproductive disorder in cattle. Embryo transfer following artificial insemination (AI) improves pregnancy rate by replenishing interferon tau (IFNT), but it results in a notably higher rate of twin occurrence. This study hypothesized that parthenogenetic (PA) embryo transfer following AI (AI + PA) could improve the conception rate because that PA embryo become as a supplemental source of IFNT without twins. PA embryos showed higher IFNT mRNA expression than *in vitro* fertilization (IVF) embryos. An examination of the effect of the cultured conditioned media (CM) of PA or IVF embryos on Madin-Darby bovine kidney cells with stably introduced promoter-reporter constructs of interferon-stimulated gene 15 (ISG15, marker of IFN response) showed higher stimulation levels of ISG15 promoter activity with PA than with IVF embryo. We investigated *in vivo* the effect of AI + PA on healthy Japanese Black cattle. Cattle transferred with PA embryo alone were non-fertile, but those that underwent AI + PA showed a pregnancy rate of 53.3%, the similar as that with AI alone (60%). In pregnant cattle in AI + PA group, adding the PA embryo upregulated the expression of ISGs and plasma progesterone concentration. No twin were generated in AI only and AI + PA groups. Using repeat breeding Holstein cows that did not become pregnant with 4–9 times of AI, transfer of PA embryo following AI resulted in a higher pregnancy rate than that of control (AI only). We suggest that AI + PA may be beneficial for improving maternal pregnancy recognition in repeat breeder cattle while avoiding twin generation.

Key words: Interferon-stimulated gene (ISG) 15, Interferon tau, Parthenogenesis, Pregnancy

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The fertility of lactating dairy cows has gradually been declining worldwide, including in Japan, and there is considerable concern about repeat breeding, one of the most critical reproductive disorders in cattle. Repeat breeders are generally described as subfertile animals without anatomical or infectious abnormalities that do not become pregnant with three or more breeding attempts, or that remain infertile after numerous services [1]. It is likely that the physiological cause of this condition is multifactorial, with considerable variation. Repeat breeder syndrome is complex, with repeat breeder cattle showing multiple endocrine dysfunctions, including of progesterone (P4), estradiol, epithelial growth factor, and luteinizing hormone [2–4].

To prevent and improve repeat breeding syndrome, many treatments aimed at regulating endocrine function have been tried and proposed. For example, improved conception rates in repeat breeder dairy cattle have reported with gonadotropin-releasing hormone, P4 formulation (controlled internal drug release), exogenous estradiol benzoate, and combinations of these [5–7]. Remarkably, embryo transfer (ET) following artificial insemination (AI) can used as an assisted reproductive technology to improve the conception rate of repeat breeder cattle [1, 3]. Similarly, the cotransfer of bovine trophoblast vesicles with *in vitro* fertilization (IVF) embryos increases the fertility of demi-embryos during the early stages of pregnancy [8, 9]. We recently reported that the transfer of IVF embryos following the AI of repeat breeder cattle resulted in the production and release of interferon tau (IFNT) [10], a well-established pregnancy recognition signal in ruminants [11], improving the conception rate. However, ET following AI resulted in a considerably higher rate of twin occurrence in repeat breeder cattle (6.25–18.4% [1, 3, 10]) than occurs during normal AI (0.3–5% in dairy cattle) [12]. There is a strong association between the occurrence of twins and the rate of labor accidents, so an alternative method that avoids twin generation is needed to improve conception in repeat breeder cattle.

Parthenogenetic (PA) embryos are those in which the oocyte develops without the male gamete, a process that does not occur

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spontaneously in mammals. During fertilization, the spermatozoon triggers multiple rhythmic oscillations of intracellular free calcium, which release the oocyte. This intracellular calcium pattern can be mimicked by a several chemicals that activate the oocyte without sperm, yielding PA embryos; these can easily be produced from slaughterhouse-derived ovaries [13, 14]. PA embryos can cause the production and secretion of IFNT in cows [15], but they are unable to develop to pregnancy in the uterus, living for a maximum of 48 days, with no fetal heartbeat [16]. Hirayama *et al*. [15] reported that the cotransfer of PA embryos with low-grade IVF embryos significantly improved the pregnancy rate on Day 40 compared with same grade of IVF embryos without PA embryos, this suggested that PA embryos provide a supplemental source of IFNT sufficient to enhance the maternal recognition of pregnancy. However, few reports have examined the influence of PA embryo transfer following AI in cows.

We hypothesized that the reproductive technique of PA embryo transfer following AI could improve the conception rate of repeat breeder cows because that PA embryo may enhance IFNT secretion while avoiding the occurrence of twins. Before conducting a large-scale field study in repeat breeder cattle, it is necessary to confirm the effectiveness and safety of PA embryo use in detail at the laboratory level. In the present study, therefore, we assessed the potential of PA embryos for IFNT production and IFN responsiveness to other cells, comparing them with IVF embryos. Then, to test the role of PA embryo *in vivo*, we investigated the effect of PA embryo transfer, with or without AI, on luteal function (plasma P4 concentrations) and the IFN response (assessed using interferon-stimulated genes (ISGs), a marker gene for IFN) in peripheral blood samples from healthy Japanese Black cows. Finally, as a field experiment, we examined the effect of PA embryo transfer following AI in repeat breeder Holstein cows that did not become pregnant even after 4–6 times or 7–9 times of 7–9 AIs.

Materials and Methods

In vitro maturation and fertilization

In vitro maturation and fertilization were performed as previously described [17]. In brief, bovine ovaries obtained from a local slaughterhouse were transported to the laboratory and the cumulus-oocyte complexes (COCs) were aspirated from the follicles (2–5 mm in diameter) and washed three times in TCM-199 (Gibco BRL, Grand Island, NY, USA) containing 20 mM HEPES supplemented with 5% fetal bovine serum (FBS; HyClone, GE Healthcare UK, Buckinghamshire, England). The COCs were matured for 20–24 h at 38.5°C in a humidified atmosphere with 5% CO_2. The matured oocytes were then inseminated with frozen-thawed semen from a Japanese Black bull (adjusted to 2×10^7 cells/ml) for 5 h at 38.5°C in a humidified atmosphere with 5% CO_2 in the air, in 1 ml of BO solution containing 10 mg/ml bovine serum albumin and 10 µg/ml heparin.

Parthenogenetic activation

Bovine oocytes were collected and matured as described above. After maturation, cumulus cells were removed from the oocytes using hyaluronidase (300 units/ml, Sigma-Aldrich, St. Louis, CA, USA). There are several ways to make PA embryos, including treatment with ethanol, calcium ionophore, and electrical stimulation. In this experiment, we chose ethanol treatment, which can be processed easy and in large amounts at one time, as we are aiming for future mass production of PA embryos [16, 18]. The oocytes were activated with 7% ethanol in phosphate-buffered saline for 1 min for two times, and then cultured in TCM-199 with 5% FBS containing 10 µg/ml cycloheximide (Sigma-Aldrich) and 5 µg/ml cytochalasin B (Sigma-Aldrich) for 6 h at 38.5°C in a humidified atmosphere with 5% CO_2 in air. The parthenogenetically activated oocytes were washed and cultured as described below.

IVF and PA embryo culture and freezing of embryos

After IVF or parthenogenic activation, oocytes were placed in fresh TCM-199 medium with 5% FBS, and the embryos were co-cultured with cumulus cells, as previously described [17]. The culture medium was changed every 2 days. At days 7 and 8, embryos that had developed into blastocysts with good-quality grade (Codes 1 or 2, classified as code 1 to 3 according to ITES manual) were stained for cell counting or collected to analysis gene expression. At least 10 expanded blastocysts of IVF and PA were fixed in 4% paraformaldehyde and fixed embryos were stained with propidium iodide for cell counting using microscope.

In addition, after 7 days, embryos with good-quality grade were frozen in 1.4 M glycerol in modified TCM-199 containing 20 mM HEPES and 0.35 mg/ml sodium bicarbonate supplemented with 20% FCS. Embryos were transferred directly into freezing medium, and each embryo was loaded into a 0.25 ml plastic straw (Fujihira, Tokyo, Japan). The straws were placed in an alcohol bath in a programmable freezer (EYELA, Tokyo, Japan) precooled to –6°C. After 1 min, the straws were seeded, maintained for another 9 min, then cooled to –25°C at a rate of –0.33°C/min and kept at 5 min before being plunged into liquid nitrogen.

RNA extraction, cDNA production, and real-time PCR

Total RNA from embryo was prepared using RNAqueous total RNA isolation kit according to the manufacturer's instructions (Thermo Fisher Scientific, Tokyo, Japan). RNA extraction and cDNA production were performed with a commercial kit (ReverTra Ace; Toyobo, Osaka, Japan). Real-time quantitative PCR was performed with the CFX Connect™ Real Time PCR system (Bio-Rad, Hercules, CA, USA) and a commercial kit (Thunderbird SYBR qPCR Mix; Toyobo) to detect the mRNA expressions of *IFNT, ISG15*, *MX dynamin like GTPase 2* (*MX2*), or *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*). The primers used for real-time PCR were as follows: forward, 5′- CATCTTCCCCATGGCCTTCG-3′ and reverse, 5′- TCATCTCAAAGTGAGTTCAG-3′ for IFNT (accession no. AF238613); forward, 5′-GGTATGATGCGAGCTGAAGCACTT-3′ and reverse, 5′-ACCTCCCTGCTGTCAAGGT-3′ for ISG15 (accession no. NM_174366); forward, 5′- GGACAGCGGAATCATCAC-3′ and reverse, 5′- CTCCCGCTTTGTCAGTTTCAG-3′ for MX2 (accession no. NM_173941); forward, 5′- ACAGTCAAGGCAGAGAACGG-3′ and reverse, 5′- CCACATACTCAGCACCAGCA-3′ for GAPDH (accession no. NM_001034034). RT-qPCR was performed in duplicate with a final reaction volume of 20 µl containing 10 µl of SYBR Green, 7.8 µl of distilled water, 0.1 µl of 100 µM forward and reverse primers, and 2 µl of cDNA template. The amplification program

consisted of a 5 min denaturation at 95ºC followed by 40 cycles of amplification (95ºC for 15 sec, 60ºC for 20 sec, and 72ºC for 10 sec). The expression levels of each target gene were normalized to the corresponding GAPDH threshold cycle (CT) values using the ΔΔ CT comparative method [19]. The relative amount of each PCR product was also calculated in comparison, using GAPDH as the international standard.

For in vitro experiment: In vitro embryo production and collection of the conditioned medium

After IVF or parthenogenic activation, oocytes surrounded by cumulus cells were placed in fresh TCM-199 medium with 5% FBS and the embryos were co-cultured with cumulus cells (100 fertilized embryos/1 ml of culture medium). After culturing for 7 days, the growth status of the embryos was confirmed, and the culture medium was collected in 3–5 independent experiments. Of these IVF culture–conditioned media (CM), there were 15.3 hatched blastocysts and 38.6 blastocyst embryos on average. Of these PA culture–CM, there were 9.2 hatched blastocysts and 41.0 blastocyst embryos on average. The CM samples (n = 3–5) were frozen and stored until the following experiment.

After culturing for 7 days, non-hatching IVF and PA expanded blastocysts (grades 1 or 2) were selected, moved to another drop (50 µl), and further cultured individually for a further day. The hatching status of each embryos was confirmed (non-hatched or hatched from the zona pellucida), and the CM of the individual embryos was collected. In total, we collected 15 samples of hatched IVF-CM, 30 samples of non-hatched IVF-CM, 6 samples of hatched PA-CM, and 46 samples of non-hatched PA-CM. These CM samples were frozen and stored until the following experiment to investigate ISG15 promoter activity.

For in vitro experiment: Preparation of splenic immune cells and treatment of embryo culture CM

To investigate the effect of CM of embryo culture on other cells, we used splenocytes as a representative example of immune cells as described previous our study [10]. In brief, splenic tissue from a local slaughterhouse was cut into small pieces, minced in PBS containing heparin and antibiotics (amphotericin B and gentamicin, Sigma-Aldrich) and centrifuged at 500 × *g* for 10 min. The pellet was resuspended and filtered (70 µm, Corning Incorporated, Corning, NY, USA). The cells were washed, treated with Ammonium–Chloride–Potassium lysing buffer (Thermo Fisher Scientific) for the lysis of red blood cells, and resuspended in RPMI 1640 medium (Life Technologies, Carlsbad, CA, USA) with 5% FBS. Next, splenic immune cells were treated with or without the CM of vehicle (cumulus cells only, without embryos), IVF embryos, and PA embryos as described above for 6 h at 38°C. After incubation, cells were collected using ISOGEN II (Nippon Gene, Tokyo, Japan) to analyze mRNA expression, and stored –80°C until analysis.

For in vitro experiment: ISG15 promoter luciferase assay

To investigate IFNT activity in the CM from the embryo culture, we used MDBK cells transfected with the ISG15 promoter-reporter vector [10, 20]. The cells were seeded at a density of 1.0×10^5 cells/well into 96-well cell culture plates using DMEM/F-12 (Sigma-Aldrich) containing antibiotics and 5% FBS for 24 h. The cells were incubated with or without the CM of vehicle (cumulus cells only, without embryos), IVF embryos, and PA embryos for 6 h. After collecting the cultured medium of MDBK cells, luciferase activity was measured using the Luciferase Reporter Assay System (Promega, Madison, WI, USA) in accordance with the manufacturer's protocol. Fluorescence was measured using a microplate reader (Spark 10M; TECAN Group, Männedorf, Switzerland).

For in vivo basic experiment: Recipient animals and PA embryo transfer

All the experimental procedures complied with the Guidelines for the Care and Use of Agricultural Animals of Tokyo University of Agriculture and all the animal protocols were approved by our institutional animal experiment committee.

Japanese Black cows in the farm of Tokyo University of Agriculture were used. All had healthy uterus and ovaries, as determined by transrectal palpation. The average age of the cows used was 6.2 years (3–12 years old), the average of body weight was 472.7 kg (420–554 kg), the average number of parturitions was 2.8 times (0–7 times), and the average of days after previous parturition was 132.2 days (30–358 days). At day 0 (estrus = day 0) of the estrous cycle, insemination (using commercially available frozen-thawed semen from Japanese Black bulls) was performed with a single straw after thawing by immersion in a water-bath at 35–38°C. On Day 7 of the estrous cycle, the PA embryo was non-surgically transferred into the uterine horn, ipsilateral to the ovary with corpus luteum. The animals were divided into three groups: Group 1 (n = 10) received AI, Group 2 (n = 4) received the transfer of a PA embryo at Day 7, and Group 3 (n = 15) received AI with the transfer of a PA embryo at Day 7. Blood samples were taken on Day 7, 14, 21, and 28 of the estrous cycle. To investigate changes in mRNA expression, whole blood was applied to Blood RNA Cards (FortiusBio LLC, San Diego, CA) following the manufacturer's protocol. The cards were dried for 2 h, then frozen at –20°C, and stored until analysis. Pregnancy was determined by transrectal palpation on Day 40 after insemination.

P4 determination

Measurement of P4 concentration in the plasma was performed using a direct enzyme immunoassay (EIA) [21]. The EIA for P4 was performed according to previous reports [21]. Within-assay and between-assay coefficients of variation of P4 were 4.7 and 6.5%, respectively.

For in vivo field experiment: Recipient animals and PA embryo transfer

Holstein cows (diagnosed as repeat breeders) from dairy farms in the east Hokkaido region of Japan were used as recipients, as defined by a previous study [1]. In brief, repeat breeder cows had the following characteristics: (1) detectable estrous behavior but occasionally abnormal estrous cycles; (2) healthy uterus and ovaries as determined by transrectal palpation; and (3) the inability to conceive after three or more inseminations following normal estrous behavior. In these repeat breeder cows, cows that did not become pregnant even after 4–6 times or 7–9 times of 7–9 AIs were used for the experiment. The PA embryo was transferred 7 or 8 days after AI

(using commercially available frozen-thawed semen from Japanese Black bulls). The PA embryo was non-surgically transferred into the uterine horn, ipsilateral to the ovary with corpus luteum. As a control, from breeding records, the data were selected that cows did not become pregnant even after 4–6 times or 7–9 times of AIs. Pregnancy was determined by transrectal palpation on days 40–60 after insemination.

Statistical analysis

All data are presented as means ± SEM. The statistical significance of differences was assessed by Student's *t*-test or one-way ANOVA followed by Bonferroni's multiple comparison test. Probabilities less than 5% ($P < 0.05$) were considered significant.

Results

Comparison of the characteristics of the IVF and PA embryos

The proportions of IVF and PA embryos that had developed to the blastocyst stage after 7 and 8 days of culturing are shown in Table 1. A significantly greater proportion of IVF embryos than PA embryos had developed to the blastocyst stage at Day 8. Representative pictures of IVF and PA embryos in culture at Day 8 are shown in Fig. 1A. The PA embryos had lower total numbers of cells than the IVF embryo (Fig. 1A). Similar to the findings previous studies, the expression of *IFNT* mRNA was significantly higher in the PA embryos than in the IVF embryos, regardless of freezing and thawing (Fig. 1B).

Comparison of IFN activity after treatment with the CM of IVF and PA embryos

To investigate our hypothesis that both types of embryos produce IFNT that affects other cells, including peripheral immune cells, we examined the effect of treating splenic immune cells *in vitro* with the CM of IVF or PA embryos or cultured vehicle CM (cumulus cells only, without embryos) as shown in Figs. 2A and 2B. The mRNA expression of both *ISG15* and *MX2* was clearly stimulated by treatment with the CM of the IVF and PA embryos. The stimulatory effect of increasing *MX2* mRNA expression was higher with the CM of the PA embryos compared to the CM of the IVF embryos (Fig. 2B).

To confirm the effect of cultured IVF and PA embryos on ISG15 transcription, we used MDBK cells with stably introduced ISG15 promoter-reporter constructs. The cells were treated with CM; they demonstrated a specific increase in reactivity with the CM of the IVF and PA embryos but not with the cultured vehicle CM (Fig. 2C). The increase in ISG15 promoter activity was higher with the CM of the PA embryos compared to the CM of the IVF embryos (Fig. 2C). These findings suggest that both IVF and PA embryos can produce and release IFNT, resulting in the stimulation of ISG15 gene expression.

Comparison of IFN activity between non-hatched and hatched blastocysts treated with the CM of IVF and PA embryos

We compared IFN activity between non-hatched embryos and those that had hatched from the zona pellucida, using separately cultured embryos. A significantly greater proportion of IVF embryos than PA embryos hatched from the zona pellucida, relative both to the number of oocytes used in the experiment or to the number of blastocyst embryos (Table 2). We then tested the effect of CM from separately cultured embryos on ISG15 promoter activity in non-hatched or hatched IVF and PA embryos (Fig. 3). Importantly, the CM of non-hatched (as well as hatched) IVF and PA embryos significantly stimulated ISG15 promoter activity. However, the CM of hatched IVF and PA embryos exhibited significantly higher IFN activity than that of non-hatched IVF and PA embryos. The IFN activity of PA embryos was significantly higher than that of IVF embryos, regardless of whether or not they had hatched from the

Table 1. Comparison of embyro development between *in vitro* fertilization (IVF) and parthenogenetic (PA) embryos

Treatments	% of blastocysts *	
	Day 7	Day 8
IVF embryo	33.3 (35/105)	38.7 [a] (74/191)
PA embryo	24.8 (165/665)	26.3 [b] (175/665)

* The number in parentheses indicate the number of oocytes.
[a, b] There is a significant difference in different shoulder letters.

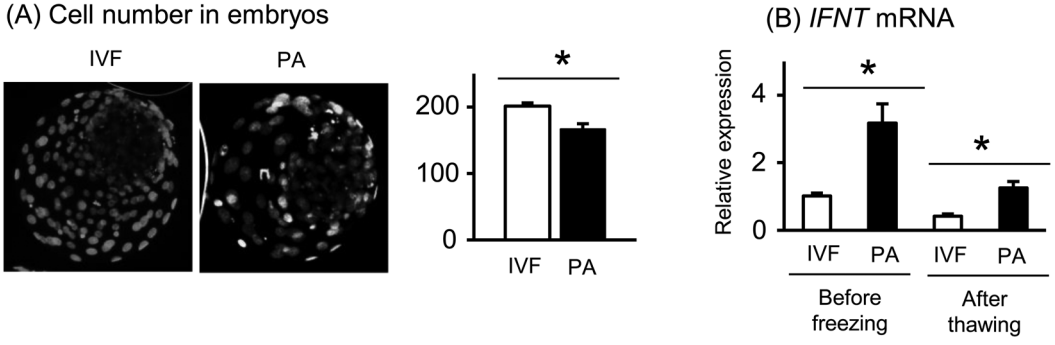

Fig. 1. Comparison of the characteristics of the IVF and PA embryos. At day 8, at least 10 expanded blastocysts of IVF and PA were fixed in 4% paraformaldehyde and cells of embryos were counted using microscope (A). Representative images of IVF and PA embryos are shown, respectively. Blastocyst embryos at day 7 were collected and the mRNA expression of IFNT (B) was determined. All values are shown as the mean ± SEM (relative to GAPDH mRNA levels). * indicates significant differences ($P < 0.05$) as determined by Student's *t*-test.

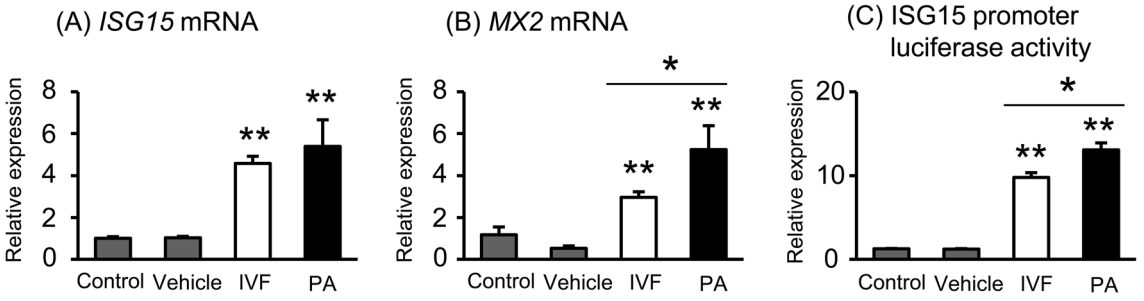

Fig. 2. Comparison of IFN activity after treatment with the CM of IVF and PA embryos. (A–B) Bovine splenic immune cells were isolated and treated with or without the CM of vehicle (cumulus cells without embryos), IVF embryos, or PA embryos for 6 h. Then, ISG15 and MX2 mRNA expression was determined (n = 3–5, each group). (C) MDBK cells transfected with the ISG15 promoter-reporter vector were used. The cells were incubated with or without the CM of vehicle (cumulus cells without embryos), IVF embryos, or PA embryos for 6 h (n = 3–5 in each treated group). After collecting the cultured medium of MDBK cells, the luciferase activity was measured using the Dual-Luciferase Reporter Assay System. * and ** indicate significant difference (P < 0.05 and 0.01) as determined by one-way ANOVA followed by Bonferroni's multiple comparison test.

Table 2. Comparison of hatched embryo between *in vitro* fertilization (IVF) and parthenogenetic (PA) embryos

Treatments	% of hatched embryo at Day 9 *	
	Data in total	Data in blastocyst
IVF embryo	26.2% [a] (50/191)	67.6% [a] (50/74)
PA embryo	8.6% [b] (57/665)	32.6% [b] (57/175)

* The number in parentheses indicate the number of oocytes.
[a, b] There is a significant difference in different shoulder letters.

zona pellucida.

Effects of PA transfer in Japanese Black cattle

A total of 29 Japanese Black cattle were used to investigate the effect of PA transfer (Table 3). The pregnancy rate in the AI group, acting as a control, was 60.0%. To check the safety of PA embryo transplantation was similar to that of ET of IVF embryos, a single PA embryo was transferred into the uterine horn on the non-corpus luteum side on Day 7 of the estrous cycle. All the cattle that underwent PA embryo transfer were non-fertile. PA embryo transfer following AI (AI + PA) resulted in a pregnancy rate of 53.3%, as similar as that for AI only. The mean estrous cycles of the cattle that were non-fertile in AI, PA, and AI + PA groups were 20.5, 26.0, and 28.4 days, respectively. Ultrasound imaging diagnostic of the uterus during days 28–40 confirmed the conceptus product in the AI (pregnancy) group, but was unable to detect it in the PA group. In all the treated groups where conception occurred, the twin rate was 0%. There was no evidence of late embryo resorption or abortion due to AI + PA, and normal labor was observed in all cows in AI + PA group.

Blood samples were collected on Days 7, 14, 21, and 28 to investigate the effect of PA transfer on plasma P4 concentrations and the mRNA expression of ISGs in white blood cells. On Day 14 (7 days after transfer), plasma P4 concentrations were higher in the AI + PA groups compared with AI and PA groups (Fig. 4A). At day 21, plasma P4 concentrations had gradually decreased in the PA and AI + PA (non pregnancy) groups, whereas P4 concentrations were maintained at higher levels in AI (pregnancy) and AI + PA (pregnancy)

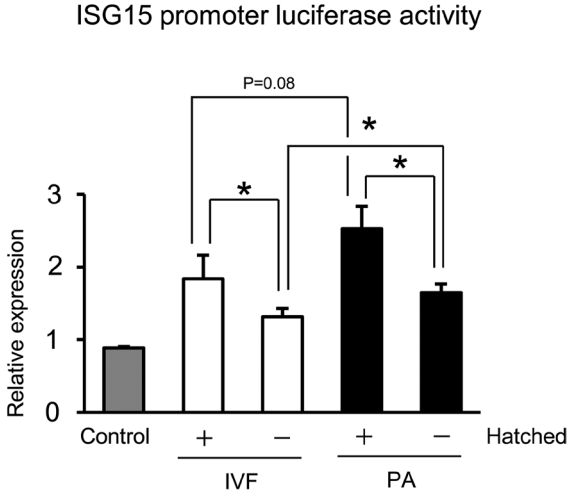

Fig. 3. Comparison of IFN activity between non-hatched and hatched blastocysts treated with the CM of IVF and PA embryos. After culturing for 7 days, non-hatching IVF and PA expanded blastocysts were selected and further cultured individually for a further day. The hatching status of each embryos was confirmed (non-hatched or hatched from the zona pellucida), and the CM of the individual embryos was collected. MDBK cells transfected with the ISG15 promoter-reporter vector were incubated with or without the CM of vehicle (cumulus cells without embryos), IVF embryos, or PA embryos for 6 h. After collecting the cultured medium of MDBK cells, the luciferase activity was measured using the Dual-Luciferase Reporter Assay System. * indicates significant difference (P < 0.05) as determined by one-way ANOVA followed by Bonferroni's multiple comparison test.

Table 3. Pregnancy and twin rate after treatment in Japanese Black cows

Treatments	No. of practice	No. of pregnancies	% of pregnancies	% of twins
AI	10	6	60	0
PA	4	0	0	0
AI + PA	15	8	53.3	0

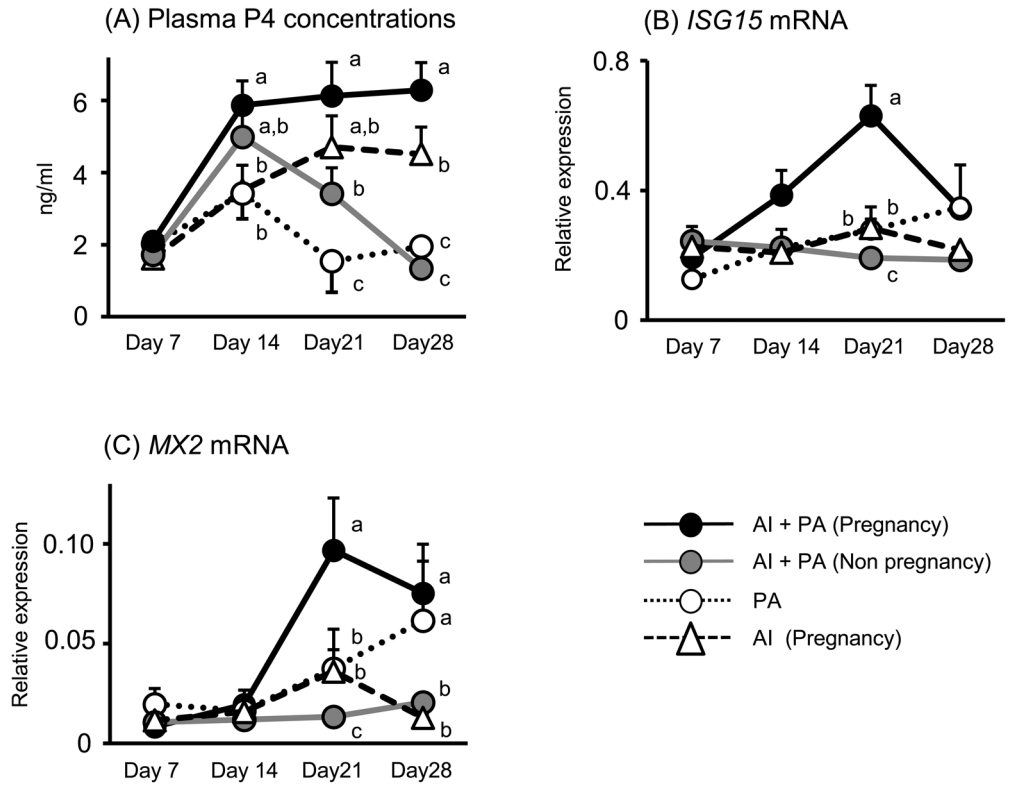

Fig. 4. Effects of PA transfer in Japanese Black cattle. Blood samples were collected on days 7, 14, 21, and 28 after estrus. Plasma P4 concentrations (A) were determined by EIA, and the mRNA expression of ISG15 (B) and MX2 (C) was determined in each group. 'a, b, c' indicates a significant difference compared to other groups in each day ($P < 0.05$), as determined by one-way ANOVA followed by Bonferroni's multiple comparison test.

groups. On Day 28, plasma P4 concentrations were significantly higher in the AI + PA (pregnancy) group than in the other groups.

Figures 4B and 4C show mRNA expression of the ISGs, such as *ISG15* and *MX2*. On Day 21, the mRNA expressions of *ISG15* and *MX2* were significantly higher in the AI (pregnancy) group than in the AI + PA (non pregnancy) group. In addition, the mRNA expressions of *ISG15* and *MX2* were further higher in the AI + PA (pregnancy) group than in other three groups. In the PA group, these *ISGs* mRNA expression gradually increased throughout the experimental phase.

Effects of PA transfer following AI in repeat breeder Holstein cow: field test

As shown in Table 4, as a control, 32.7% in repeat breeder Holstein cows (603/1843 cows) were pregnant with 4–6 times of AI and 29.4% in repeat breeder Holstein cows (158/536 cows) were pregnant with 7–9 times of AI. In the final investigation, we examined the effect of PA transfer following AI in 95 repeat breeder Holstein cows. Transfer of PA embryo to cows that did not become pregnant with 4–6 times of AI resulted in a pregnancy rate of 37.9% (22/58 cows), which was tended to be higher than that of control (AI only). In repeat breeder Holstein cows that did not become pregnant with 7–9 times of AI, transfer of PA embryo also significantly increased a pregnancy rate of 40.5% (15/37 cows) than that of control (29.4%).

Discussion

In the present study, we first investigated the characteristic of PA embryos. Hatching from the zona pellucida is a prerequisite for the preimplantation embryo to attach to the uterus. After hatching from the zona pellucida, blastocyst stage embryos develop into elongated

Table 4. Pregnancy rate after treatment in repeat breeding Holstein cows

Treatments	No. of AI: 4–6 times		No. of AI: 7–9 times	
	No. of practice	No. of pregnancies (%)	No. of practice	No. of pregnancies (%)
AI	1843	603 (32.7)	536	158 (29.4) [a]
AI + PA	58	22 (37.9)	37	15 (40.5) [b]

embryos, with increasing secretion of IFNT. Conversely, it has been reported that Day 7 bovine non-hatched embryos secrete IFNT in the uterus, regulating the uterine microenvironment *in vivo* [22]. Using the described PA embryo production method, the production rate of the blastocyst stage of the PA embryos and the rate of hatching from the zona pellucida were significantly low compared with IVF embryos. The following three points are suggested as reasons for this; (1) Because of the lower total number of cells in the PA embryos, it is possible that there was insufficient physical pressure for hatching from the zona pellucida. Indeed, the cell cycle length of the PA embryos appeared to be longer than that of the IVF embryos [23]. PA embryos produced by ethanol activation (a method similar to that of the present study) have few cells [24], and the apoptotic indexes of PA embryos are higher than that of IVF embryos [25]. (2) It is possible that the PA embryos had only a small amount of the enzyme that melts the zona pellucida. It has been reported that urokinase-type plasminogen activator is the dissolution enzyme for the zona pellucida in cattle [26]. (3) Hardening of the zona pellucida may occur differently in PA embryos than in other embryos. Koester *et al*. [27] reported that the rate of hatching from the zona pellucida in PA embryos (37%) was lower than that in IVF embryos (52%) and that the birefringence intensity of the zona pellucida was associated with blastocyst formation and hatching ability. On the other hand, it is thought that the total number of cells and embryo development change according to the PA embryo production method. For example, other PA embryo production methods, including ionomycin and 6-dimethylaminopurine, resulted in a higher total cell number and blastocyst production rate than IVF embryos, with the same rate of hatching from the zona pellucida [13, 28].

Next, we examined the potential roles of PA embryos in IFNT production and IFN responsiveness to other cells, comparing the IVF embryos. As in previous reports, the mRNA expression of *IFNT* was significantly higher in PA embryos than in IVF embryos [14, 28]. Moreover, using CM of PA and IVF embryos, we demonstrated that CM of PA embryo, with or without hatching from the zona pellucida, stimulated ISG activity to a greater extent than did the CM of IVF embryo, indicating the higher productive capacity of PA embryo for IFNT. Yao *et al*. [29] reported that *IFNT* mRNA began to be expressed at the 16-cell stage in IVF embryos and at the blastocyst stage in PA embryos. Importantly, Rashid *et al*. [22] demonstrated that non-hatching blastocyst embryos can produce and secrete IFNT, inducing anti-inflammatory response in the bovine uterus. Recently, we showed that the transfer of IVF embryos following AI has the potential to increase the IFNT response, improving conception rates in repeat breeder cattle [10]. These findings suggest that a PA embryo supplementation effect can be expected in order to complement IFNT in repeat breeder cattle.

The combined treatment with ET following AI provides an effective therapeutic tool for the improvement of fertility in repeat breeder cattle via the addition of IFNT [1, 3]. However, this method can result in the generation of embryos, and the increase in the incidence of twin pregnancies with this method is of concern. Therefore, we hypothesized that PA embryo transfer could potentially result in IFNT secretion while avoiding twins, and in this study we tested our hypothesis *in vivo* using healthy Japanese Black cows. This confirmed the safety of PA embryo transfer; no twins occurred and no adverse effects were observed from PA embryo transfer following AI. Similar to our previous study, we confirmed in the pregnancy groups that levels of *ISGs* mRNA expression had increased in peripheral white blood cells at Day 21 after PA embryo transfer following AI compared with AI alone (Fig. 4). Whether or not pregnancy occurred, plasma P4 concentrations were tended to be higher with PA embryo transfer following AI than with AI alone (pregnancy) on Day 14 and, in the pregnancy group of PA embryo transfer following AI, they continued at higher levels throughout the period to Day 28. Hirayama *et al*. [15] demonstrated that PA embryos secrete IFNT and that the inhibition of luteolysis by transfer of PA embryos resulted in maintained high levels of plasma P4 concentrations in cows. In living bovine organisms, IFNT produced by the conceptus passes through the endometrium and enters the uterine vein [30], playing a crucial role in the transformation of tissues during pregnancy, including the corpus luteum [31]. Unfortunately, IFNT does not have a role to regulate P4 production directly [32]. On the other hand, IFNT stimulates neutrophil chemoattractant interleukin-8 (IL-8) and accumulates neutrophils within the corpus luteum [33]. Moreover, both IFNT-activated neutrophils and IL-8 stimulate P4 secretion from luteal cells in vitro, suggesting the potential role of IFNT for acceleration of P4 secretion from the corpus luteum. These findings and observations suggest that adding PA embryo following AI would provide a supplemental source of IFNT sufficient to support and establish pregnancy in cattle.

In the present study, PA embryo transfer has no effect on conception rate in normal fertile Japanese black cattle. It is thought this was because of the use of Japanese Black cattle with a high conception rate (60% in the present study) compared with the normal conception rate in Holstein cows, or because the hatching rate of PA embryos was low. On the other hand, the scale of field experiment is not so large, but we showed that the transplantation test of PA embryo following AI resulted in the improvement of conception rate in repeat breeder Holstein cow. Therefore, it is possible that PA embryo transfer can improve fertility in repeat breeder Holstein cow with poor reproductive condition, but not normal reproductive cows. In our preliminary experiment, we confirmed that PA embryos produced in the present study were able to hatch from the zona pellucida *in vivo*, as were the IVF embryos. However, we recently reported that as a result of IVF embryo transfer following AI, pregnancy rates were higher at 44.8% in repeat breeder Holstein cows compared with the present result of PA embryo transfer following AI. These findings indicate that PA embryos produced in the present study need to be improved to achieve higher pregnancy rates in repeat breeder cows. In future, we intend to investigate an alternative method for the production of PA embryos to improve the hatching rate from the zona pellucida to further increase the effect of IFNT *in vivo*.

In conclusion, the present study revealed that bovine PA embryo at blastocyst stage has a higher potential to secrete IFNT than IVF embryo, with a low development rate of blastocyst production and hatching from the zona pellucida. We confirmed that the added PA embryo produces and secretes IFNT, resulting in the increased expression of ISGs and maintained higher levels of plasma P4 in cattle. In repeat breeder Holstein cows, transfer of PA embryo following AI resulted in a higher pregnancy rate than that of control (AI only). Thus, the reproductive technique of PA embryo transfer

following AI may be beneficial for improving conception in repeat breeder cattle while avoiding twin generation. Future investigation are needed to examine the potential improvement role of PA embryo transfer in the conception rate of repeat breeder cattle in a large-scale field study and its mechanisms *in vivo*.

Conflict of interest: The authors declare no conflict of interest.

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